Mechanobiological aspects of (dysregulated) wound healing and the foreign body response

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CHAPTER 1

INTRODUCTION AND AIM OF THIS THESIS

INTRODUCTION

Multicellular organisms are made up out of smaller building blocks called cells. In 1847, Schleiden and Schwann concluded that cells are the common building blocks of tissues, both in plants as well as in animals[1]. Rapid developments in the field of cell biology in the mid-20th century enabled investigators to culture metazoan cells outside of the living organism. Cell culturing in vitro - which literally means "on glass" - was performed on glass or polystyrene, while these materials have a stiffness that exceeds the physiological stiffness range found in the body by orders of magnitude (Figure 1).

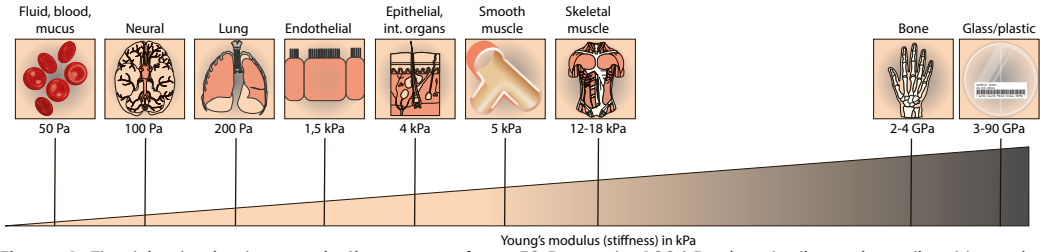

Figure 1. The biophysical range in tissue goes from 50 Pa up to 100 kPa (excluding mineralized bone). Glass and plastic are in the GPa range, with at least a 100,000x higher Young's modulus than found in vivo[2].

Most cells cultured in vitro are anchorage dependent, which means that they need to adhere to be able to survive and carry out their function. However, the chemical and physical properties of the substrate the cells adhere to can have profound effects on cell behavior. Mechano-biologists are at the interface of engineering and biology, and focus on the effect physical properties and other mechanical forces have on the cell. Physical properties include ligand density[3], rheology[4], topographic changes[5] and stiffness[6–8]. Mechanical forces include (but are not limited to) tension[9,10], compression[11], shear stress[12], and strain[13]. How cells sense these mechanical forces is called mechanosensing, while the conversion of mechanical signals into a biochemical response is called mechanotransduction. Mechanical forces can influence tissue development, cell behavior, as well as the progression and outcome of diseases[14]. As such, cellular mechanosensing and mechanotransduction play pivotal roles in a wide range of intricate biological and (patho)physiological processes[2,15]. In this thesis we will mainly focus on the effect

of stiffness (or elastic modulus) on the phenotypical properties and gene expression profiles of macrophages and fibroblasts in wound repair, fibrosis and the foreign body reaction[16–18].

TENSEGRITY

Several methods and models have been developed to better understand the effect of mechanical forces on cell biology[7,19,20]. In the late 90's of the past century Donald E. Ingber adopted the tensegrity model (first introduced by Buckminster Fuller in the field of architecture) to better understand cellular mechanosensing[21,22]. Ingber's tensegrity model states that the cell cytoskeleton is in a continuous state of isometric tension and therefore stabilizes the cells' morphology. Changes in isometric tension, for example due to changes in substrate stiffness[17], can have profound effects on the cells' morphology[23–25] and behavior[26,27]. For instance, fibroblasts mimic the stiffness of their underlying substrate in order to reduce intercellular isometric tension[17] and this stiffness mimicking leads to a decrease in cell size on softer substrates. Decrease in cell size is accompanied by a loss of alpha smooth muscle actin, which means fibroblasts lose their contractile phenotype[28,29].

Ingber posit tensegrity as the best fitting model for cellular structure based on the observation that cells flatten on a solid stiff surface but become spherical when non-adherent (Figure 2), as observed after trypsin treatment[21,22,30]. Most scientists studying mechanobiology have adopted tensegrity as the best fitting model for cellular mechanosensing[11,22,30].

THE CYTOSKELETON, FOCAL ADHESIONS AND INTEGRINS

Mechanotransduction signals are transduced via changes in the cytoskeleton - a complex network of interlinking filaments consisting of actin and tubulin - and these changes directly regulate cell morphology and movement. In terms of tensegrity the actin filaments exert a constant inward-force creating tension between the cell membrane and the nucleus, while constant compression is created by tubulin and specialized anchoring receptors - such as clustered integrins at focal adhesions[21]. The

balance (or equilibrium) between this compression and tension is the basis of cellular tensegrity[11,22,30].

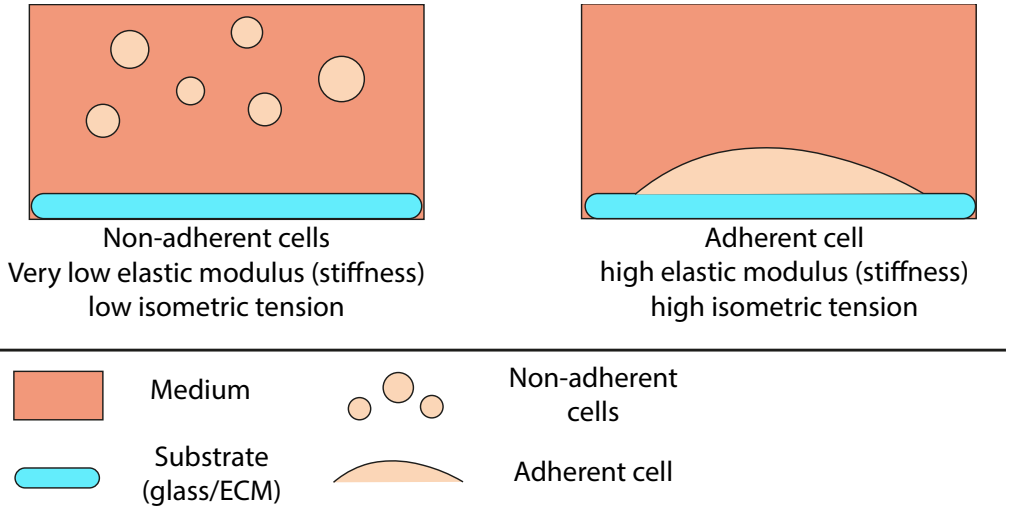

Figure 2. Differences in isometric tension based on cellular adherence. Non-adherent cells become rounded, while adherent cells become flat and spread more. Differences in elastic modulus (Young's modulus or stiffness) lead to a similar effect in most cells: the lower the stiffness, the more rounded and smaller the cells become.

Cells are surrounded in vivo by a fibrous mixture of extracellular molecules that provides mechanical and biochemical support, also known as the extracellular matrix (ECM). The ECM is largely deposited by a specific subset of cells of mesenchymal origin called fibroblasts, and consists of fibrous proteins (e.g. collagen, fibronectin), polysaccharides (glycosaminoglycans, hyaluronic acid) and water.

In adherent cells, focal adhesions tether the ECM that surrounds and supports cells to the cytoskeleton via integrins. Integrins are heterodimers consisting of a wide variety of α- and β-subunit combinations, and expression of each subunit is highly dependent on ECM type. Although we will not focus on integrins in detail, it is important to understand the basic principles of cellular adhesion to the ECM via these calcium-dependent receptors to understand general mechanosensing.

Integrins reside at the cell-ECM interface and exert external forces onto linker proteins in focal adhesion complexes. Talin and paxillin, being the most abundant linker proteins in focal adhe-

sion complexes, recruit focal adhesion kinase (FAK) and vinculin. Phosphorylation of a-actinin by FAK leads to a-actinin and vinculin adhesion and crosslinks and tethers actomyosin (F-actin and non-muscle myosin II complex) stress fibers to the cytoplasmic membrane via focal adhesion sites[31]. Adaptor protein p130Cas and the membrane-associated tyrosine kinase Src regulate integrin clustering in a stiffness-dependent manner (Figure 3). Recently, an SH3 (Src kinase homolog 3) domain in all-spectrins, a family member of a-actinin, has been linked to initial integrin clustering and cell spreading as well[32]. In Chapter 5 we studied the effect of stiffness on spectrins in fibroblasts. In Chapter 2 we report on the effect of stiffness on cell morphology and size using hydrogels that were coated with ECM molecules via a novel cross-link procedure. Furthermore, we studied in the same setting the effect of stiffness on vinculin, a mechanosensing protein in focal adhesions[19,20].

STIFFNESS AND CELL DEVELOPMENT

Stiffness plays an import role in cell development and differentiation[33]. In a seminal study by Engler et al., mesenchymal stem cells cultured on soft (1 kPa), intermediate (12 kPa) or stiff (>20 kPa) substrates obtained different morphologies and differentiated into functionally different cell types due to stiffness alone[8]. Soft substrates led to differentiation into an adipogenic phenotype, intermediate stiffness induced a myogenic phenotype while a stiff (rigid) substrate could induce an osteogenic phenotype[8,34,35]. Many different cell types have been studied for their mechanosensitivity, and it has even been speculated that all prokaryotic and eukaryotic cells offer some form of mechanosensing due to the presence of mechanosensitive ion channels[36]. Indeed, changes in differentiation state, proliferation[31], migration[37,38] and morphology[17,23,28] have been observed in a wide variety of cells due to changes in substrate stiffness leading to changes in intercellular isometric tension[17].

Mechanosensing describes how cells feel the physical properties of their surroundings. Consecutively these mechanical signals can be converted into biochemical activity, a process called mechanotransduction[7,21,39–41]. For example, transcription factor

translocation (e.g. Yes-associated protein, YAP) can be induced via mechanotransduction pathways, thereby inducing cell differentiation and/or proliferation[15,42–45]. These data suggest that chemical and mechanical signaling cues are equally important in cell differentiation and cellular response.

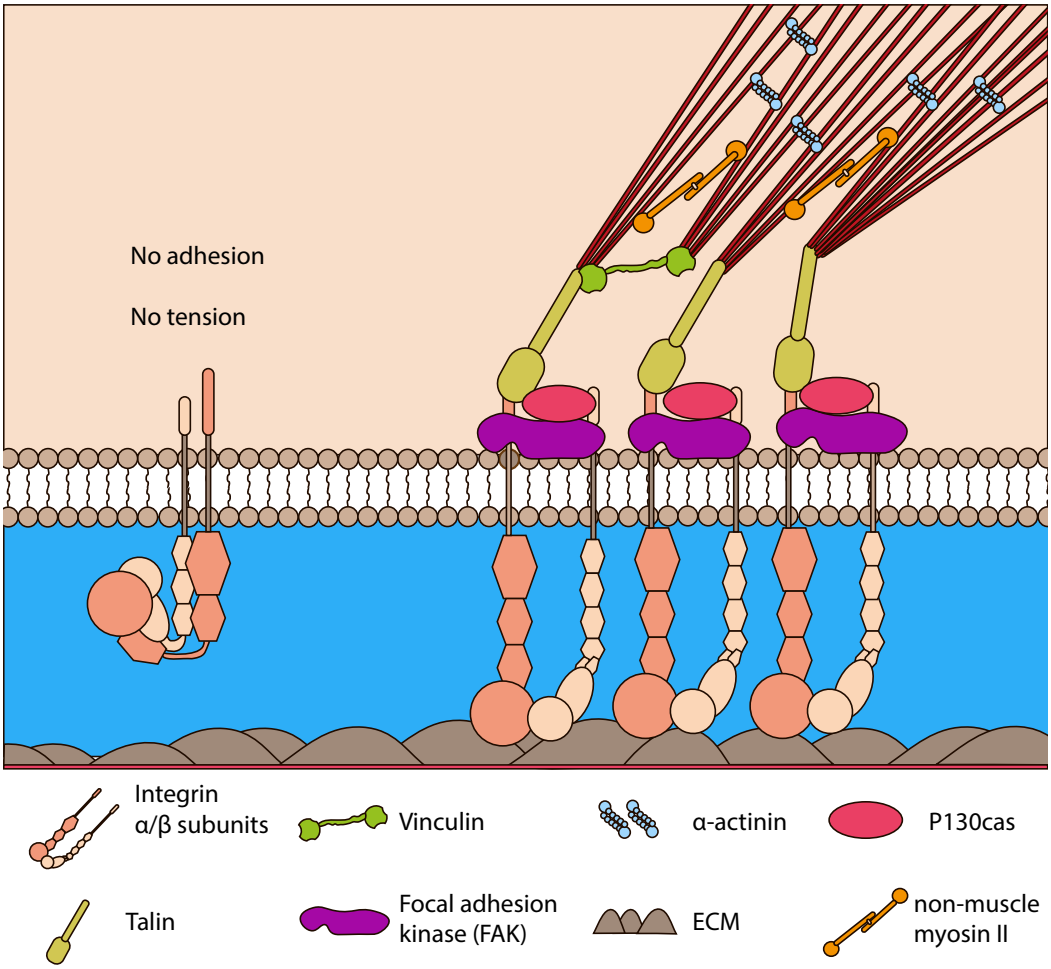

Figure 3. Integrins and focal adhesions in cell-substrate adherence. Integrins are membrane spanning calcium-dependent adhesion receptors, which link the ECM to the intracellular cytoskeleton and therefor transduce mechanical signals to the cell.

THE EXTRACELLULAR MATRIX: WOUND REPAIR

In non-pathological conditions, fibroblasts are involved in tissue homeostasis and ECM remodeling, effectively balancing ECM synthesis and breakdown. During wound repair, and in response

to trauma, platelets and polymorphonuclear neutrophils (PMNs) extravasate from locally damaged blood vessels and infiltrate the area of the insult. During the coagulation phase platelets coagulate with fibrin to form the hemostatic plug at the wound site, and start to release transforming growth factor β1 (TGFβ1) and platelet derived growth factor (PDGF). TGFβ1 is an important driver of fibrosis and much effort has been put into limiting the effects of TGFβ1. Although PMNs are the most abundant inflammatory cells during early inflammation (24 h) they are replaced by macrophages at the late inflammatory phase (48 h). Macrophages and PMNs play an important role in cellular debris phagocytosis and remodeling of damaged tissue and newly synthesized ECM. Influx of T-cells and mast cells leads to secretion of large quantities of the M2 inducing cytokines IL4 and IL13. These cytokines induce native marcophages and blood derived macrophage to differentiate towards an anti-inflammatory (wound healing) M2 phenotype. Later we will see that these M2 inducing cytokines also induce macrophage multinucleation.

As fibroblasts are attracted to the wound site, they differentiate into myofibroblasts under the influence of TGFβ1. Myofibroblasts are profibrotic cells with a highly contractile phenotype. In vivo, fibroblasts and myofibroblasts are in direct and continuous contact with the ECM, and mechanical force plays an important role in fibroblast to myofibroblast differentiation and activation of latent TGFβ1.

As fibroblasts differentiate into myofibroblasts, an equilibrium shift occurs towards ECM production. More collagen type I is produced and at the same time this collagen type I is modified and cross-linked, which leads to an attenuation of collagen degradation. Myofibroblasts sense the cross-linked collagen and further contract the collagen fiber network to close the wound, which leads to tissue stiffening. In normal wound healing collagens are remodeled in the weeks after wound closure, which leads to a decrease in myofibroblasts, and subsequently shifts the ECM production back towards homeostasis equilibrium.

FIBROSIS

Fibrosis is defined as the accumulation of excessive fibrous connective tissue due to an aberrant and dysregulated wound healing response[46,47]. Myofibroblasts, key players in fibrosis, are usually positive for a-smooth muscle actin (ACTA2) and have an acquired capacity to synthesize collagen I, the main component of the fibrotic ECM. A forward feedback loop exists in fibrosis in which the stiffened ECM is both a cause and consequence of (myo)fibroblasts activation.

Where in normal wound healing collagen synthesis is attenuated after wound closure, an imbalance in collagen synthesis and degradation exists during fibrosis, and the fibrillary collagens are extensively cross-linked, further attenuating degradation. Fibrosis leads to an increased tissue stiffening, replacing healthy tissue with excessive scar formation. While most tissues in vivo range between 0.1 and 20 kPa, fibrotic tissues can reach up to 100 kPa, which can have profound effects on all cells found in the damaged tissue, both mesenchymal as well as hematopoetic.

Fibroblast function is largely dependent on ascorbic acid - which has it name derived from scurvy (ascorbic = anti scorbutic agent), as it regulates collagen deposition. In Chapter 7 we further elucidated the synergistic/cofounding role of ascorbic acid on fibroblasts function and myofibroblast formation.

FOREIGN BODY RESPONSE AND THE FIBROUS CAPSULE

Biomaterials are used on a daily basis in the clinic, e.g. for cosmetic purposes, reconstructive surgery, tissue engineering, and implantation of sensors[48]. Our body is tuned in such a way that it will elicit a material-dependent inflammatory response to most foreign body objects. This response is called the foreign body response (FBR)[49]. During the FBR macrophages remain at the implant surface, and can elicit macrophage fusion, resulting in multinucleated giant cells[50]. During the remodeling phase fibroblasts form a fibrous capsule (or foreign body granuloma) around the biomaterial, while macrophages mainly reside at the biomaterial interface. Macrophages are captured between the dense fibrous capsule and the implant, and syncytia called foreign body

giant cells (FBGCs) are formed[51,52]. Not much is known about the role and function of these FBGCs, but they can reside for over a decade at the implant surface, and microscopic cracks in the biomaterials have been observed under FBGCs.

In the past decade, research focused onto limiting the FBR and formation of the fibrous capsule, mainly by modulating the topography and surface chemistry of the biomaterial[53–56]. During fibrous capsule formation excess collagen type I deposition, cross-linking and contraction by (myo)fibroblasts introduces changes in the physiological stiffness, which might cause a forward feedback loop, thereby influencing cell behavior. On the other hand, implanted biomaterials themselves often introduce non-physiological stiffness and we know from both in vivo and in vitro studies that this non-physiological stiffness can regulate and orchestrate cell signaling and cell response, ultimately modulating cell fate.

We wondered whether changes in stiffness, as found in wound healing, fibrosis and the FBR, might influence macrophage polarization and behavior, much like it influences fibroblast behavior. Macrophages play an important role during the FBR in a variety of ways. For instance, macrophages secrete cytokines and growth factors which directly alter fibroblast function. Moreover, macrophages can infiltrate and, depending on biomaterial properties, degrade the biomaterial by phagocytosis and/or secretion of matrix metalloproteinases and/or other enzymes.

As our understanding of biomaterials and mechanobiology progresses it becomes more and more evident that in order to regulate the FBR, we need to regulate three pillars; the first pillar being the state of the cell of interest, the second is (to control release of) soluble factors (growth factors, cytokines, chemokines) and the third encompasses the properties (both chemical and mechanical) of the direct (micro)environment of the cell. For the third pillar we need to modulate and tightly control the properties of the biomaterial (e.g. the structure, organization, topography, stiffness and ligand density).

AIM OF THIS THESIS

Although many different forces are exerted on cells in vivo, the aim of this thesis is to identify how cells respond to changes in substrate stiffness in a wound healing / fibrotic / foreign body setting, without convolution of stiffness, surface chemistry and ECM density. Every organ in the body has a different approximate stiffness (Figure 1) which can be measured with different techniques, e.g. by atomic force microscopy. The physiological range of healthy organs ranges from 0.1 to 20 kPa and cells in these organs respond to changes in stress and strain due to stiffness changes. These changes can directly influence cell morphology and behavior. To put things in perspective: most in vitro studies thus far have been performed on polystyrene (PS) with an approximate stiffness 100,000 times higher compared to dermal tissue. Yet for decades' researchers expected (and still expect!) cells adhered to a stiff substrate to respond in a native way to cytokines and growth factors, and make direct comparisons with the in vivo situation. In this thesis we aimed to develop a novel system to study the effect of substrate stiffness on the cells regulating wound repair, fibrosis and the FBR: the fibroblasts and macrophages.

DESIGN AND RATIONALE

In **Chapter 2** we developed a reliable, robust and reproducible hydrogel system for in vitro cell culture using polyacrylamide (PAAm) which can be used to study 2D mechanosensing pathways. We used 3,4-dihydroxy-L-phenylalanine (L-DOPA) as a novel cross-linker to adhere ECM components to the otherwise inert polyacrylamide hydrogels to enable cell cultures. L-DOPA outperforms sulfo-SANPAH cross-linking (the golden standard) of ECM to PAAm in every way. ECM functionalization was more homogeneous, stable and more efficient. Furthermore, L-DOPA is more economical and can quite easily be applied by even basic laboratories, as it does not need fancy equipment or chemistry knowledge. To test our model, we used fibroblasts and observed effects similar to studies performed on either sulfo-SANPAH coated PAAMs or PDMS. Cell size and the number of vinculin-rich focal adhesion complexes were increased in a stiffness-dependent manner.

In **Chapter 3** we used our newly developed PAAm in vitro culturing system to study the effect of stiffness on macrophage polarization when cultured on collagen type I.

In **Chapter 4** we investigated the effect of stiffness in combination with fibronectin coating on the formation of macrophage-derived foreign body multinuclear giant cells.

In **Chapter 5** we investigated the CD44 pathway regarding stiffness-based macrophage fusion.

In **Chapter 6** we studied the role of spectrins in wound healing and mechanosensing in the key wound healing cell, the fibroblast.

In **Chapter 7** we studied the effect of ascorbic acid on fibroblast behavior and myofibroblast formation.

Concluding remarks, discussion and future prospects are presented in **Chapter 8**, where an overview linking all studies can be found.

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